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(54) Title: XYLANASE VARIANTS

(57) Abstract: The present invention relates to a variant xylanase polypeptide, or fragment thereof having xylanase activity, comprising one or more amino acid modifications such that the polypeptide or fragment thereof has an altered sensitivity to a xylanase inhibitor and has an altered thermo-sensitivity as compared with the parent xylanase enzyme.

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XYLANASE VARIANTS

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Field of the invention

10 The present invention relates to mutant xylanase enzymes having an altered sensitivity to xylanase inhibitors as well as altered thermo-sensitivity. The present invention also relates to the use of these mutant enzymes in processing plant materials.

Background to the invention

15 For many years, endo- β -1,4-xylanases (EC 3.2.1.8) (referred to herein as xylanases) have been used for the modification of complex carbohydrates derived from plant cell wall material. It is well known in the art that the functionality of different xylanases (derived from different microorganisms or plants) differs enormously, as a function of pH and temperature. Based on these characteristics, different xylanases have been
20 chosen for different industrial applications. Xylanases selected for the baking industry tend to be mesophilic regarding thermostability and temperature optima. Thus, they have activity at dough processing temperatures (typically 20-40°C) and they are inactivated during baking. In the feed industry, xylanases with different temperature characteristics are preferred. Optimally, xylanases for use in the feed industry are
25 thermostable to retain activity after heat treatment and pelleting. Furthermore, it has recently been demonstrated, that inhibitors of xylanases may influence the functionality and efficiency of xylanases. However, combining these characteristics in variant polypeptides is not reported, and may be used to obtain tailored and more efficient xylanases.

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Summary of the invention

The present invention relates to more useful xylanases and methods for obtaining same.

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- 5 The data presented herein show that xylanase inhibitors dictate the functionality and efficiency of the xylanases currently used in, for example, wheat based systems. Furthermore, it is also shown that changing the xylanases sensitivity towards xylanase inhibitors, can be combined with changed thermo-sensitivity of the xylanase. Such a combination makes it possible to tailor a xylanase with the right functionality and
- 10 thermo-sensitivity

Furthermore, we have gone on to design and test a series of xylanases modified by site-directed mutagenesis to demonstrate that xylanases can be produced that have a reduced sensitivity to xylanase inhibitors present in plant material and changes thermo-

15 sensitivity.

In this context, thermo-sensitivity means the xylanases' thermostability and/or temperature optimum. Both characteristics known to persons skilled in the art.

- 20 In particular, we have identified a number of residues in family 11 xylanases which influence the degree of inhibition of the xylanase and a number of residues which influence the thermo-sensitivity.

Thus, it will be possible to produce variant xylanases having a modified (for example

25 reduced) sensitivity to xylanase inhibitors and hence altered functionality and a modified thermo-sensitivity.

Altering the functionality, for example thermo-sensitivity and inhibitor sensitivity of a xylanase, will, for example, allow a reduction in the amount of xylanase required in a

30 number of applications such as animal feed, starch production, bakery, flour separation (wetmilling), and paper and pulp production. In addition or alternatively, altering the functionality of a xylanase may make it possible to ensure that xylanase activity is inactivated at moderate, desirable temperatures, without using costly and often highly detrimental temperature elevations in the material processed.

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The present invention provides a method of altering the sensitivity of a xylanase polypeptide to an inhibitor and of altering the thermo-sensitivity of said xylanase polypeptide, which method comprises modifying, preferably modifying one or more amino acid residues of, a parent xylanase polypeptide to provide a variant xylanase polypeptide, and testing the sensitivity of said variant xylanase polypeptide to a xylanase inhibitor compared with the parent xylanase enzyme and testing the thermosensitivity of said variant xylanase polypeptide compared with the parent xylanase enzyme and, optionally, selecting a variant xylanase polypeptide having an altered sensitivity to a xylanase inhibitor and an altered thermo-sensitivity compared with the parent xylanase enzyme.

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The present invention further provides a variant xylanase polypeptide, or fragment thereof having xylanase activity, comprising one or more amino acid modifications such that the polypeptide or fragment thereof has an altered sensitivity to a xylanase inhibitor compared with a parent enzyme and has an altered thermo-sensitivity as compared with a parent enzyme.

10

The term "parent enzyme" as used herein means an xylanase enzyme from which the variant xylanase enzyme is derived or derivable. With respect to the term "derivable", the variant need not necessarily be derived from the parent enzyme. Instead, the variant could be prepared, for example, by use of recombinant DNA techniques that utilise nucleotide sequence(s) encoding said variant xylanase sequence - i.e. here the nucleotide sequence(s) are similar to mutated nucleotide sequence(s) but they are not prepared by mutation of the parent nucleotide sequence(s). The variant may even be prepared by chemically modifying a parent enzyme. For some embodiments the parent enzyme is the wild type enzyme. The term "wild type" is a term of art understood by skilled persons and includes a phenotype that is characteristic of most of the members of a species occurring naturally and contrasting with the phenotype of a mutant. Thus, in the present context, the wild type enzyme may be a form of the enzyme naturally found in most members of the relevant species. Generally, the relevant wild type enzyme in relation to the variant polypeptides of the invention is the most closely

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5 related corresponding wild type enzyme in terms of sequence homology. For example, for the particular mutant xylanases described in the examples, the corresponding wild type enzyme is the wild type *Bacillus subtilis* xylanase A, more specifically the wild type *B. subtilis* xylanase A published by Paice *et al.*, 1986 and shown as SEQ ID No. 1. However, where a particular wild type sequence has been used as the basis for
10 producing a variant polypeptide of the invention, this will be the corresponding wild type sequence regardless of the existence of another wild type sequence that is more closely related in terms of amino acid sequence homology. The parent may even be a naturally occurring mutant of the wild type. In a highly preferred aspect the polypeptide of the present invention is prepared by mutating (e.g. by use of biological
15 and/or chemical techniques) a parent enzyme. For some embodiments, preferably the variant polypeptide is derived from a family 11 xylanase.

By way of example, the *B. subtilis* amino acid sequence for xylanase A is shown as SEQ ID No. 1. With respect to this sequence, the amino acid residues giving altered
20 xylanase inhibitor sensitivity may be one or more of:

D11, G12, G13, H5, N17, N29, S31, N32, G34, Y113, N114, D119, G120,
D121, R122, T123, T124 and Q175

25 In a further aspect, with respect to the *B. subtilis* amino acid sequence of xylanase A as shown in SEQ ID No. 1, the amino acid residues giving altered xylanase inhibitor sensitivity may be one or more of:

D11, G12, G13, G34, D121, R122 and T124

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The mutations giving altered thermo-sensitivity may be the mutations mentioned below or combinations thereof in respect of the *B. subtilis* amino acid sequence for xylanase A as shown in SEQ ID No. 1:

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5 S2A, N8Y, I15M, S22P, N29T, S31Q, T44V, F48N, T50V, V57I, A59E,
N61S, S74N, P75A, P90A, T93N, K99N, T111M, D121T, T123Q, T126Q,
Y128F, A142V, T143S, T147S, K154R, H156K, N163S, M169L, S179R.

By choosing an appropriate combination of mutations, for example from the lists
10 above, a xylanase with a desired inhibitor sensitivity and thermo-sensitivity can be
obtained. Thus, the inhibitor sensitivity and thermo-sensitivity of the xylanase can be
individually controlled.

Thus, preferably a variant xylanase polypeptide, or fragment thereof having xylanase
15 activity according to the present invention has an amino acid modification at any one
or more of the amino acid residues: 2, 8, 11, 12, 13, 15, 17, 22, 29, 31, 32, 34, 44, 48,
50, 57, 59, 61, 74, 75, 90, 93, 99, 111, 113, 114, 119, 120, 121, 122, 123, 124, 126,
128, 142, 143, 147, 154, 156, 163, 169, 175 and 179, or combinations thereof, based
on the amino acid numbering of *B. subtilis* xylanase shown as SEQ ID No. 1, or the
20 equivalent residues in other homologous xylanase polypeptides.

Preferably a variant xylanase polypeptide, or fragment thereof having xylanase activity
according to the present invention has at least one amino acid modification at any one
or more of the amino acid residues 11, 12, 13, 15, 17, 29, 31, 32, 34, 113, 114, 119,
25 120, 121, 122, 123, 124, and 175 together with at least one amino acid modification to
any one or more of the amino acid residues 2, 8, 15, 22, 29, 31, 44, 48, 50, 57, 59, 61,
74, 75, 90, 93, 99, 111, 121, 123, 126, 128, 142, 143, 147, 154, 156, 163, 169 and 179
based on the amino acid numbering of *B. subtilis* xylanase shown as SEQ ID No. 1, or
the equivalent residues in other homologous xylanase polypeptides.

30 Suitably, a variant xylanase polypeptide, or fragment thereof having xylanase activity
according to the present invention may have an amino acid modification at any one or
more of amino acid residues: 11, 12, 13, 15, 17, 22, 29, 31, 32, 34, 44, 48, 50, 57, 59,
61, 74, 75, 90, 93, 99, 111, 113, 114, 119, 120, 121, 123, 124, 126, 128, 142, 143, 147,
35 154, 156, 163, 169 and 179, or combinations thereof, based on the amino acid

- 5 numbering of *B. subtilis* xylanase shown as SEQ ID No. 1, or the equivalent residues in other homologous xylanase polypeptides.

Suitably a variant xylanase polypeptide, or fragment thereof having xylanase activity according to the present invention may have at least one amino acid modification at
10 any one or more of the amino acid residues 11, 12, 13, 15, 17, 29, 31, 32, 34, 113, 114, 119, 120, 121, 123 and 124 together with at least one amino acid modification to any one or more of the amino acid residues 15, 22, 29, 31, 44, 48, 50, 57, 59, 61, 74, 75, 90, 93, 99, 111, 121, 123, 126, 128, 142, 143, 147, 154, 156, 163, 169 and 179 based on the amino acid numbering of *B. subtilis* xylanase shown as SEQ ID No. 1, or
15 the equivalent residues in other homologous xylanase polypeptides.

By way of example only, suitable combinations of mutations may include:

- D11F with one or more of S2A, N8Y, I15M, S22P, N29T, S31Q, T44V, F48N, T50V, V57I, A59E, N61S, S74N, P75A, P90A, T93N, K99N, T111M, D121T, T123Q,
20 T126Q, Y128F, A142V, T143S, T147S, K154R, H156K, N163S, M169L, S179R;

- D11F and G13F, with additionally one or more of S2A, N8Y, I15M, S22P, N29T, S31Q, T44V, F48N, T50V, V57I, A59E, N61S, S74N, P75A, P90A, T93N, K99N, T111M, D121T, T123Q, T126Q, Y128F, A142V, T143S, T147S, K154R, H156K,
25 N163S, M169L, S179R;

- D11F and G34D, with additionally one or more of S2A, N8Y, I15M, S22P, N29T, S31Q, T44V, F48N, T50V, V57I, A59E, N61S, S74N, P75A, P90A, T93N, K99N, T111M, D121T, T123Q, T126Q, Y128F, A142V, T143S, T147S, K154R, H156K,
30 N163S, M169L, S179R; or

- D11F and R122D, with additionally one or more of S2A, N8Y, I15M, S22P, N29T, S31Q, T44V, F48N, T50V, V57I, A59E, N61S, S74N, P75A, P90A, T93N, K99N, T111M, D121T, T123Q, T126Q, Y128F, A142V, T143S, T147S, K154R, H156K,
35 N163S, M169L, S179R.

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The present invention further relates to a variant xylanase obtainable by, preferably obtained by, either directly or indirectly the method according to the present invention.

10 In a further embodiment, the present invention relates to a further variant xylanase prepared from a variant xylanase obtainable by, preferably obtained by, carrying out the method of the present invention.

Preferably, the inhibitor is an inhibitor found naturally in plant tissues. Suitably the inhibitor may be a *Triticum aestivum* xylanase-inhibitor (TAXI). Preferably the
15 sensitivity of the variant xylanase enzyme to the inhibitor is reduced as compared to the parent xylanase enzyme.

Preferably, the thermostability of the variant xylanase enzyme is increased.

20 Preferably, the thermostability of the variant xylanase enzyme is increased by at least 5%, more preferably by at least 10%, even more preferably by at least 15%, even more preferably by at least 20%, even more preferably by at least 30%, even more preferably by at least 40%, even more preferably by at least 50% relative to the parent enzyme when determined using the "Thermostability assay" detailed hereinbelow.

25

Preferably, the thermosensitivity of the variant xylanase is decreased.

Preferably, the thermosensitivity of the variant xylanase enzyme is decreased by at least 5%, more preferably by at least 10%, even more preferably by at least 15%, even
30 more preferably by at least 20%, even more preferably by at least 30%, even more preferably by at least 40%, even more preferably by at least 50% relative to the parent enzyme.

Preferably, the variant xylanases have a reduced sensitivity to xylanase inhibitors.

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- 5 Preferably, the sensitivity of the variant xylanase enzyme to a xylanase inhibitor is reduced by at least 5%, more preferably by at least 10%, even more preferably by at least 15%, even more preferably by at least 20%, even more preferably by at least 30%, even more preferably by at least 40%, even more preferably by at least 50% relative to the parent enzyme when determined using the "Xylanase inhibitor assay" detailed
10 hereinbelow.

- The present invention also provides a nucleic acid molecule (a nucleotide sequence) encoding a polypeptide of the invention. Also provided is a vector comprising a nucleic acid of the invention, optionally operably linked to a regulatory sequence
15 capable of directing expression of said nucleic acid in a suitable host cell. A host cell comprising a nucleic acid or a vector of the invention is also provided.

- In another aspect the present invention provides a method of making a polypeptide of the invention comprising transforming a host cell with a nucleic acid encoding said
20 polypeptide, culturing the transformed cell and expressing said polypeptide.

- Our results show that these variant polypeptides have improved properties that make them suitable for a variety of applications, such as bakery, animal feed, starch production, flour separation (wetmilling) and, paper and pulp production.
25

Accordingly, the present invention also provides the use of a variant polypeptide of the invention in a method of modifying plant materials.

- Also provided is the use of a variant polypeptide of the invention in baking. The
30 invention further provides the use of a variant polypeptide of the invention in processing cereals, starch production and animal feed and the use of a variant polypeptide of the invention in processing wood, for example in enhancing the bleaching of wood pulp.

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- 5 The invention further provides a dough that is obtainable by, preferably obtained by, the use of the variant polypeptide and baked products that are obtained by baking such a dough, and noodle and pasta products prepared in accordance with the invention.

Accordingly, the invention further relates to a method of preparing a flour dough, said
10 method comprising adding to the dough components a variant xylanase in accordance with the present invention.

In a further aspect, there is provided a dough improving composition comprising a
variant xylanase according to the present invention.

15 In the same way that variant xylanases may be used in a variety of processing applications, xylanase inhibitors may be used in a variety of processing applications such as bakery, wood pulp processing and cereal processing.

20 Detailed description of the invention

Although in general any molecular techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989) and Ausubel *et al.*, Short Protocols in Molecular Biology
25 (1999) 4th Ed, John Wiley & Sons, Inc.

A. Variant xylanase polypeptides

- 30 Xylanase enzymes have been reported from nearly 100 different organisms, including plants, fungi and bacteria. The xylanase enzymes are classified into several of the more than 40 families of glycosyl hydrolase enzymes. The glycosyl hydrolase enzymes, which include xylanases, mannanases, amylases, β -glucanases, cellulases and other carbohydrases, are classified based on such properties as the sequence of

- 5 amino acids, the three dimensional structure and the geometry of the catalytic site (Gilkes, et al., 1991, Microbiol. Reviews 55: 303-315).

Of particular interest for baking applications are the enzymes classified in Family 11. All of these are xylanases and are known as the "Family 11 xylanases". Some
 10 publications refer to these synonymously as the Family G xylanases, but the term "Family 11 xylanases" will be used herein to refer to both Family G and Family 11 xylanases.

Table A lists a number of known Family 11 xylanases. Most of them have a molecular
 15 mass of about 21,000 Da. Three of the Family 11 xylanases (*Clostridium stercoararium* XynA, *Streptomyces lividans* XynB, and *Thermomonospora fusca* XynA) have a higher molecular mass of 31,000 to 50,000 Da. However, these xylanases have a catalytic core sequence of about 21,000 Da similar to the other Family 11 xylanases. The amino acid sequences of the Family 11 xylanases (or, for the larger enzymes, the
 20 catalytic core) show a high degree of similarity, usually with more than 40 % identical amino acids in a proper amino acid alignment. The Family 11 xylanases, which are of bacterial, yeast, or fungal origin, share the same general molecular structure.

Figure 1 shows amino acid sequence alignment data in respect of 51 Family 11
 25 xylanases.

TABLE A - Family 11 xylanases

<i>Aspergillus niger</i> Xyn A	<i>Aspergillus kawachii</i> Xyn C
<i>Aspergillus tubigenensis</i> Xyn A	<i>Bacillus circulans</i> Xyn A
<i>Bacillus pumilus</i> Xyn A	<i>Bacillus subtilis</i> Xyn A
<i>Cellulomonas fimi</i> Xyn D	<i>Chainia</i> spp. Xyn
<i>Clostridium acetobutylicum</i> Xyn B	<i>Clostridium stercoararium</i> Xyn A

<i>Fibrobacter succinogenes</i> Xyn C	<i>Neocallimastix patriciarum</i> Xyn A
<i>Nocardiopsis dassonvillei</i> Xyn II	<i>Ruminococcus flavefaciens</i> Xyn A
<i>Schizophyllum commune</i> Xyn	<i>Streptomyces lividans</i> Xyn B
<i>Streptomyces lividans</i> Xyn C	<i>Streptomyces</i> sp. No. 36a Xyn
<i>Streptomyces thermoviolaceus</i> Xyn II	<i>Thermomonospora fusca</i> Xyn A
<i>Trichoderma harzianum</i> Xyn	<i>Trichoderma reesei</i> Xyn I
<i>Trichoderma reesei</i> Xyn II	<i>Trichoderma viride</i> Xyn

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Variant xylanases of the invention

A variant xylanase polypeptide of the invention is typically obtained by modifying a xylanase polypeptide by substituting, deleting or adding one or more amino acid residues within the amino acid sequence of the xylanase polypeptide. Preferably the modification comprises one or more amino acid substitutions. Modification of polypeptide sequences can be carried out using standard techniques such as site directed mutagenesis. The modification may also occur by chemical techniques – such as chemical modification of one or more amino acid residues.

15

The starting sequence may be a wild type sequence or a non-naturally occurring sequence, for example a derivative that has already been subjected to protein engineering. The xylanase sequence to be modified may be from any source, for example a bacterial, fungal or plant source. Preferably the xylanase sequence to be modified is that of a Family 11 xylanase, more preferably a Family 11 xylanase selected from *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma harzianum* xylanase, *Trichoderma viride* xylanase, *Bacillus circulans* xylanase A, *Bacillus subtilis* xylanase A, *Bacillus stearothermophilus* xylanase A, *Aspergillus niger* xylanase A, *Aspergillus kawachii* xylanase C, *Aspergillus tubigenensis* xylanase A, *Streptomyces lividans* xylanase B, and *Streptomyces lividans* xylanase C.

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- 5 In a particularly preferred embodiment, the xylanase sequence to be modified is the *B. subtilis* xylanase sequence shown as SEQ ID No. 1 or a homologue thereof. Preferably said homologue has at least 40, 50, 60 or 80% homology over at least 50 or 100 amino acid residues as determined using the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.*, 1984, Nucleic Acids Research
10 12:387).

- Specific modifications that are possible according to the present invention include one or more amino acid substitutions at positions 11, 12, 13, 15, 17, 29, 31, 32, 34, 113, 114, 119, 120, 121, 122, 123, 124 and 175 regarding inhibitor sensitivity and one or
15 more substitutions at positions 2, 8, 15, 22, 29, 31, 44, 48, 50, 57, 59, 61, 74, 75, 90, 93, 99, 111, 121, 123, 126, 128, 142, 143, 147, 154, 156, 163, 169 and 179 regarding thermo-sensitivity, or combinations thereof based on the amino acid numbering of *B. subtilis* xylanase shown as SEQ ID No. 1, or the equivalent residues in other homologous xylanase polypeptides.

- 20 Possible substitutions include one or more of D11→Y, D11→N, D11→F, D11→K, D11→S, D11→W, G12→F, G13→F, I15→K, N17→K, N17→Y, N17→D, N29→K, N29→Y, N29→D, S31→K, S31→Y, S31→D, N32→K, G34→D, G34→F, G34→T, Y113→A, Y113→D, Y113→K, N114→A, N114→D, N114→F, N114→K,
25 D119→K, D119→Y, D119→N, G120→K, G120→D, G120→F, G120→Y, G120→N, D121→N, D121→K, D121→F, D121→A, R122→D, R122→F, R122→A, T123→K, T123→Y, T123→D, T124→K, T124→Y, T124→D, Q175→E, Q175→S and Q175→L regarding inhibitor sensitivity (with reference to the amino acid sequence of *B. subtilis* xylanase) or their equivalents in other homologous xylanase
30 polypeptides and one or more substitutions of S2→A, N8→Y, I15→M, S22→P, N29→T, S31→Q, T44→V, F48→N, T50→V, V57→I, A59→E, N61→S, S74→N, P75→A, P90→A, T93→N, K99→N, T111→M, D121→T, T123→Q, T126→Q, Y128→F, A142→V, T143→S, T147→S, K154→R, H156→K, N163→S, M169→L, S179→R regarding thermo-sensitivity (with reference to the amino acid sequence of *B. subtilis* xylanase) or their equivalents in other homologous xylanase polypeptides and
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5 combinations thereof. Further references to specific residues of the *B. subtilis* xylanase shown as SEQ ID No. 1 will also include their equivalents in other homologous xylanase polypeptides.

A combination of mutations may be carried out, for example mutations at two or more,
10 three or more, four or more, five or more, ten or more, or fifteen or more of the above-mentioned residues.

In a further embodiment, the variant polypeptides of the invention may be purified and isolated naturally occurring mutant xylanases. Alternatively, mutant xylanases may be
15 generated by subjecting organisms to mutagens and then screening for individuals comprising mutations in their xylanase genes. Naturally occurring mutants and mutants generated by random mutagenesis may be identified/screened using a variety of techniques such as PCR screening using suitable nucleic acid primers to amplify regions of xylanase genes and sequencing the resulting fragments.

20 Thus variant polypeptides of the invention include naturally occurring mutant xylanases (purified and isolated from the organisms in which they occur or obtained recombinantly), mutant xylanases obtained by random mutagenesis and mutant xylanases obtained by site-directed mutagenesis.

25 Variant polypeptides of the invention may also be subjected to further modifications that do not necessarily affect sensitivity to inhibitors or thermo-sensitivity, including any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acids from or to the sequence providing the resultant
30 amino acid sequence retains xylanase activity, preferably having at least substantially the same xylanase activity as the unmodified sequence.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in
35 the third column may be substituted for each other:

5

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

Polypeptides of the invention also include fragments of the full-length sequences mentioned above having xylanase activity.

- 10 Polypeptides of the invention may further comprise heterologous amino acid sequences, typically at the N-terminus or C-terminus, preferably the N-terminus. Heterologous sequence may include sequences that affect intra or extracellular protein targeting (such as leader sequences).
- 15 Polypeptides of the invention are typically made by recombinant means, for example as described below. However they may also be made by synthetic means using techniques well known to skilled persons such as solid phase synthesis. Polypeptides of the invention may also be produced as fusion proteins, for example to aid in extraction and purification. It may also be convenient to include a proteolytic cleavage
- 20 site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences, such as a thrombin cleavage site. Preferably the fusion protein will not hinder the function of the protein sequence of interest.

- The use of appropriate host cells is expected to provide for such post-translational
- 25 modifications as may be needed to confer optimal biological activity on recombinant expression products of the invention.

- 5 Polypeptides of the invention may be in a substantially isolated form. It will be understood that the protein may be mixed with carriers or diluents which will not interfere with the intended purpose of the protein and still be regarded as substantially isolated. A polypeptide of the invention may also be in a substantially purified form, in which case it will generally comprise the protein in a preparation in which more
10 than 90%, e.g. 95%, 98% or 99% of the protein in the preparation is a polypeptide of the invention.

- Variant polypeptides of the invention have an altered sensitivity to xylanase inhibitors and an altered thermo-sensitivity compared to the parent xylanase sequence – which
15 may be a corresponding wild type xylanase. Preferably, variant polypeptides have reduced sensitivity to xylanase inhibitors and reduced thermo-sensitivity. The term “altered sensitivity to xylanase inhibitors” means that the extent to which the endo- β -1,4-xylanase activity of a variant polypeptide of the invention is inhibited by the xylanase inhibitor is different to that of the parent xylanase enzyme – which may be
20 the corresponding wild type xylanase. Preferably the extent to which the variant polypeptide is inhibited by the inhibitor is less than that of the parent xylanase enzyme – which may be the wild type protein. This may, for example, be due to a change in the three-dimensional structure of the variant polypeptide such that the inhibitor no longer binds with the same affinity as it does to the parent xylanase enzyme – which may be
25 the wild type enzyme. The term “altered thermo-sensitivity” means that the variant polypeptide of the invention has an altered thermostability or temperature optimum determined as described in the methods included below. This may, for example, be due to a change which increases the denaturing temperature of the polypeptide, i.e. that temperature at which the peptide is denatured, compared to the parent xylanase enzyme
30 – which may be the wild type enzyme.

- The sensitivity of the variant polypeptides of the invention to xylanase inhibitors can be assayed using the xylanase inhibitor assay described below. A suitable inhibitor for use in the assay is the inhibitor purified from wheat flour in Example 1. Other
35 inhibitors are described below. The thermo-sensitivity of the variant polypeptide of the

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- 5 invention may be described using the thermostability and temperature optimum assay described below.

Xylanase assay (Endo- β -1,4-Xylanase activity)

- 10 Xylanase samples are diluted in citric acid (0.1 M) - *di*-sodium-hydrogen phosphate (0.2 M) buffer, pH 5.0, to obtain approx. OD = 0.7 in the final assay. Three dilutions of the sample and an internal standard with a defined activity are thermostated for 5 minutes at 40°C. At time = 5 minutes, 1 Xylazyme tab (crosslinked, dyed xylan substrate) is added to the enzyme solution. At time = 15 minutes (or in some cases
15 longer, depending on the xylanase activity present in the sample) the reaction is terminated, by adding 10 ml of 2% TRIS. The reaction mixture is centrifuged and the OD of the supernatant is measured at 590 nm. Taking into account the dilutions and the amount of xylanase, the activity (TXU, Total-Xylanase-Units) of the sample can be calculated relative to the standard.

20

Xylanase inhibitors

- As used herein, the term "xylanase inhibitor" refers to a compound, typically a protein, whose role is to control the depolymerisation of complex carbohydrates, such as
25 arabinoxylan, found in plant cell walls. These xylanase inhibitors are capable of reducing the activity of naturally occurring xylanase enzymes as well as those of fungal or bacterial origin. Although the presence of xylanase inhibitors have been reported in cereal seeds (see for example McLauchlan et al 1999a; Rouau and Suget 1998) their impact on the efficacy of xylanase enzymes has not been extensively examined.

30

- McLauchlan et al (1999a) disclose the isolation and characterisation of a protein from wheat that binds to and inhibits two family-11 xylanases. Likewise, WO 98/49278 demonstrates the effect of a wheat flour extract on the activity of a group of microbial xylanases all of which are classified as family 11 xylanases. Debyser et al. (1999) also
35 disclose that endoxylanases from *Aspergillus niger* and *Bacillus subtilis*, which are both members of the family 11 xylanases were inhibited by a wheat xylanase inhibitor

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- 5 called TAXI (*Triticum aestivum* xylanase-inhibitor). McLauchlan *et al* (1999b) teach that extracts from commercial flours such as wheat, barley, rye and maize are capable of inhibiting both family 10 and 11 xylanases.

The xylanase inhibitor may be any suitable xylanase inhibitor. By way of example, the
10 xylanase inhibitor may be the inhibitor described in WO-A-98/49278 and/or the xylanase inhibitor described by Rouau, X. and Surget, A. (1998), McLauchlan, R., et al. (1999b) and/or the xylanase inhibitor described in International Patent Publication No. WO00/39289.

- 15 In some applications, preferably the inhibitor is a TAXI inhibitor. As indicated above, the TAXI inhibitor is disclosed in, for example Debyser et al (1999) and/or WO00/39289. An example TAXI inhibitor may comprise one or more of the amino acid sequences presented as SEQ ID No's 13-19 in WO00/39289.

- 20 The inhibitor may be one or more inhibitors of the same type, for example TAXI. For some applications, the inhibitor may be a mixture of inhibitors of different types, for example TAXI and XIP (Xylanase inhibitor proteins).

Xylanase inhibitor assay

- 25 100 µl of an candidate inhibitor fraction, 250 µl xylanase solution (containing 12 TXU microbial xylanase/ml) and 650 µl buffer (0.1 M citric acid - 0.2M *di*-sodium hydrogen phosphate buffer, pH 5.0) are mixed. The mixture is thermostated for 5 minutes at 40.0°C. At time = 5 minutes one Xylazyme tab is added. At time = 15 minutes the
30 reaction is terminated by adding 10 ml 2% TRIS. The reaction mixture is centrifuged (3500 g, 10 minutes, room temperature) and the supernatant is measured at 590 nm. The inhibition is calculated as residual activity compared to the blank. The blank is prepared the same way, except that the 100 µl inhibitor is substituted with 100 µl buffer (0.1 M citric acid - 0.2 M *di*-sodium hydrogen phosphate buffer, pH 5.0).

35

5 Specific xylanase inhibitor

As indicated, a xylanase inhibitor that may be used in accordance with the present invention is the xylanase inhibitor described in International Patent Publication No. WO00/39289.

10

This endogenous endo- β -1,4-xylanase inhibitor is obtainable from wheat flour. The inhibitor is a di-peptide, having a MW of about 40 kDa (as measured by SDS-PAGE or mass spectrometry) and a pI of about 8 to about 9.5.

- 15 Sequence analysis to date has revealed that the inhibitor has at least one or more of the sequences presented as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7 and/or SEQ ID No. 8.

- These inhibitors described in the prior art may also be used in assays to determine the sensitivity of a variant polypeptide of the invention to xylanase inhibitors. They may also be used as described below to modulate the functionality of a xylanase.
- 20

Thermostability assay

- 25 Solutions of the variant polypeptide are incubated for 10 to 240 minutes, preferably 30 to 240 minutes, at temperatures varying from 30 to 80°C, preferably at 50, 55, 60 or 65°C. Hereafter the xylanase solutions are assayed as described in "Xylanase assay" above. Activities as a function of different pre-incubation temperatures are expressed relatively to the highest activity obtained.

30

Temperature optimum assay.

- Solutions of the variant polypeptides are assayed as described in "Xylanase assay", except for the temperature. Instead of only assaying the xylanase variants at 40°C, the xylanases may be incubated and assayed at one or more of the following temperatures:
- 35

- 5 30, 40, 50, 60 and 70°C. Hereby it is possible to determine a temperature optimum for the xylanase variants.

Polynucleotides

- 10 Polynucleotides of the invention comprise nucleic acid sequences encoding the variant polypeptide sequences of the invention. It will be understood by a skilled person that numerous different polynucleotides can encode the same polypeptide as a result of the degeneracy of the genetic code. In addition, it is to be understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the
- 15 polypeptide sequence encoded by the polynucleotides of the invention to reflect the codon usage of any particular host organism in which the polypeptides of the invention are to be expressed.

- Polynucleotides of the invention may comprise DNA or RNA. They may be single-stranded or double-stranded. They may also be polynucleotides which include within
- 20 them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be
- 25 understood that the polynucleotides described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of polynucleotides of the invention.

Nucleotide vectors and host cells

- 30 Polynucleotides of the invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a
- 35 replicable vector, introducing the vector into a compatible host cell, and growing the

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5 host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells include bacteria such as *E. coli*, yeast and fungi.

Preferably, a polynucleotide of the invention in a vector is operably linked to a
10 regulatory sequence which is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding
15 sequence is achieved under conditions compatible with the control sequences. The term "regulatory sequences" includes promoters and enhancers and other expression regulation signals.

Enhanced expression of the polynucleotide encoding the polypeptide of the invention
20 may also be achieved by the selection of heterologous regulatory regions, e.g. promoter, secretion leader and terminator regions, which serve to increase expression and, if desired, secretion levels of the protein of interest from the chosen expression host and/or to provide for the inducible control of the expression of the polypeptide of the invention.

25 Aside from the promoter native to the gene encoding the polypeptide of the invention, other promoters may be used to direct expression of the polypeptide of the invention. The promoter may be selected for its efficiency in directing the expression of the polypeptide of the invention in the desired expression host.

30 In another embodiment, a constitutive promoter may be selected to direct the expression of the desired polypeptide of the invention. Examples of strong constitutive and/or inducible promoters which are preferred for use in fungal expression hosts are those which are obtainable from the fungal genes for xylanase (*xlnA*), phytase, ATP-synthetase, subunit 9 (*oliC*), triose phosphate isomerase (*tpi*), alcohol
35

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- 5 dehydrogenase (*AdhA*), α -amylase (*amy*), amyloglucosidase (AG - from the *glcA* gene), acetamidase (*amdS*) and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoters.

- Examples of strong yeast promoters are those obtainable from the genes for alcohol
10 dehydrogenase, lactase, 3-phosphoglycerate kinase and triosephosphate isomerase.

Examples of strong bacterial promoters are the α -amylase and *SP02* promoters as well as promoters from extracellular protease genes.

- 15 Hybrid promoters may also be used to improve inducible regulation of the expression construct.

- Often, it is desirable for the polypeptide of the invention to be secreted from the expression host into the culture medium from where the polypeptide of the invention
20 may be more easily recovered. According to the present invention, the polypeptide of the invention's native secretion leader sequence may be used to effect the secretion of the expressed polypeptide of the invention. However, an increase in the expression of the polypeptide of the invention sometimes results in the production of the protein in levels beyond that which the expression host is capable of processing and secreting,
25 creating a bottleneck such that the protein product accumulates within the cell. Accordingly, the present invention also provides heterologous leader sequences to provide for the most efficient secretion of the polypeptide of the invention from the chosen expression host.

- 30 According to the present invention, the secretion leader may be selected on the basis of the desired expression host. A heterologous secretion leader may be chosen which is homologous to the other regulatory regions of the expression construct. For example, the leader of the highly secreted amyloglucosidase (AG) protein may be used in combination with the amyloglucosidase (AG) promoter itself, as well as in

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- 5 combination with other promoters. Hybrid signal sequences may also be used with the context of the present invention.

Examples of preferred heterologous secretion leader sequences are those originating from the fungal amyloglucosidase (AG) gene (*glaA* - both 18 and 24 amino acid versions e.g. from *Aspergillus*), the α -factor gene (yeasts e.g. *Saccharomyces* and *Kluyveromyces*) or the α -amylase gene (*Bacillus*).

- Such vectors may be transformed into a suitable host cell as described above to provide for expression of a polypeptide of the invention. Thus, in a further aspect the invention provides a process for preparing polypeptides according to the invention which comprises cultivating a host cell transformed or transfected with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the polypeptides, and recovering the expressed polypeptides. Suitable host cells include, for example, fungal cells, such as *Aspergillus* and yeast cells, such as yeast cells of the genus *Kluyveromyces* or *Saccharomyces*. Other suitable host cells are discussed below.

- The vectors may be for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes. The most suitable selection systems for industrial micro-organisms are those formed by the group of selection markers which do not require a mutation in the host organism. Examples of fungal selection markers are the genes for acetamidase (*amdS*), ATP synthetase, subunit 9 (*oliC*), orotidine-5'-phosphate-decarboxylase (*pvrA*), phleomycin and benomyl resistance (*benA*). Examples of non-fungal selection markers are the bacterial G418 resistance gene (this may also be used in yeast, but not in fungi), the ampicillin resistance gene (*E. coli*), the neomycin resistance gene (*Bacillus*) and the *E. coli uidA* gene, coding for β -glucuronidase (GUS). Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell.

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A further embodiment of the invention provides host cells transformed or transfected with a polynucleotide of the invention. Preferably said polynucleotide is carried in a vector for the replication and expression of said polynucleotides. The cells will be chosen to be compatible with the said vector and may for example be prokaryotic (for
10 example bacterial), fungal, yeast or plant cells.

15

Bacteria from the genus *Bacillus* are very suitable as heterologous hosts because of their capability to secrete proteins into the culture medium. Other bacteria suitable as hosts are those from the genera *Streptomyces* and *Pseudomonas*.

Depending on the nature of the polynucleotide encoding the polypeptide of the invention, and/or the desirability for further processing of the expressed protein, eukaryotic hosts such as yeasts or fungi may be preferred. In general, yeast cells are preferred over fungal cells because they are easier to manipulate. However, some
20 proteins are either poorly secreted from the yeast cell, or in some cases are not processed properly (e.g. hyperglycosylation in yeast). In these instances, a fungal host organism should be selected.

A heterologous host may also be chosen wherein the polypeptide of the invention is
25 produced in a form which is substantially free from other xylanases. This may be achieved by choosing a host which does not normally produce such enzymes.

Examples of preferred expression hosts within the scope of the present invention are fungi such as *Aspergillus* species and *Trichoderma* species; bacteria such as *Bacillus*
30 species, *Streptomyces* species and *Pseudomonas* species; and yeasts such as *Kluyveromyces* species and *Saccharomyces* species.

Particularly preferred expression hosts may be selected from *Aspergillus niger*, *Aspergillus niger* var. *tubigenis*, *Aspergillus niger* var. *awamori*, *Aspergillus*
35 *aculeatis*, *Aspergillus nidulans*, *Aspergillus oryzae*, *Trichoderma reesei*, *Bacillus*

- 5 *subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Kluyveromyces lactis*,
 Lactococcus lactis, *Thermomyces (Humicola) lanuginosus*, *Pichia* spp.,
 Schizosaccharomyces pombe, *Mucor* spp. and *Saccharomyces cerevisiae*.

According to the present invention, the production of the polypeptide of the invention
10 can be effected by the culturing of microbial expression hosts, which have been
 transformed with one or more polynucleotides of the present invention, in a
 conventional nutrient fermentation medium.

The fermentation medium can comprise a known culture medium containing a carbon
15 source (e.g. glucose, maltose, molasses, etc.), a nitrogen source (e.g. ammonium
 sulphate, ammonium nitrate, ammonium chloride, etc.), an organic nitrogen source
 (e.g. yeast extract, malt extract, peptone, etc.) and inorganic nutrient sources (e.g.
 phosphate, magnesium, potassium, zinc, iron, etc.). Optionally, an inducer may be
 added.

20 The selection of the appropriate medium may be based on the choice of expression
 hosts and/or based on the regulatory requirements of the expression construct. Such
 media are well known to those skilled in the art. The medium may, if desired, contain
 additional components favouring the transformed expression hosts over other
25 potentially contaminating microorganisms.

After fermentation, the cells can be removed from the fermentation broth by means of
centrifugation or filtration. After removal of the cells, the variant polypeptide of the
invention may then be recovered and, if desired, purified and isolated by conventional
30 means.

Organisms

The term "organism" in relation to the present invention includes any organism that
35 could comprise the nucleotide sequence coding for the variant xylanase protein according

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5 to the present invention and/or products obtained therefrom, wherein a transcriptional regulatory sequence can allow expression of the nucleotide sequence according to the present invention when present in the organism. Suitable organisms may include a prokaryote, fungus, yeast or a plant. For the xylanase aspect of the present invention, a preferable organism may be a bacterium, preferably of the genus *Bacillus*, more
10 preferably *Bacillus subtilis*.

The term "transgenic organism" in relation to the present invention includes any organism that comprises the nucleotide sequence coding for the protein according to the present invention and/or products obtained therefrom, wherein the transcriptional
15 regulatory sequence can allow expression of the nucleotide sequence according to the present invention within the organism. Preferably the nucleotide sequence is incorporated in the genome of the organism.

The term "transgenic organism" does not cover native nucleotide coding sequences in
20 their natural environment when they are under the control of their native promoter which is also in its natural environment.

Therefore, the transgenic organism of the present invention includes an organism comprising any one of, or combinations of, the nucleotide sequence coding for the amino
25 acid sequence according to the present invention, constructs according to the present invention (including combinations thereof), vectors according to the present invention, plasmids according to the present invention, cells according to the present invention, tissues according to the present invention or the products thereof. The transformed cell or organism could prepare acceptable quantities of the desired compound which would
30 be easily retrievable from, the cell or organism.

Transformation of Host Cells/Host Organisms

As indicated earlier, the host organism can be a prokaryotic or a eukaryotic organism.
35 Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*. Teachings

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- 5 on the transformation of prokaryotic hosts is well documented in the art, for example see Sambrook et al (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press) and Ausubel *et al.*, Short Protocols in Molecular Biology (1999), 4th Ed., John Wiley & Sons, Inc.
- 10 If a prokaryotic host is used then the nucleotide sequence may need to be suitably modified before transformation - such as by removal of introns.

As mentioned above, a preferred host organism is of the genus *Bacillus*, such as *Bacillus subtilis*.

- 15 In another embodiment the transgenic organism can be a yeast. In this regard, yeasts have also been widely used as a vehicle for heterologous gene expression. The species *Saccharomyces cerevisiae* has a long history of industrial use, including its use for heterologous gene expression. Expression of heterologous genes in *Saccharomyces*
- 20 *cerevisiae* has been reviewed by Goodey et al (1987, Yeast Biotechnology, D R Berry et al, eds, pp 401-429, Allen and Unwin, London) and by King et al (1989, Molecular and Cell Biology of Yeasts, E F Walton and G T Yarronton, eds, pp 107-133, Blackie, Glasgow).
- 25 For several reasons *Saccharomyces cerevisiae* is well suited for heterologous gene expression. First, it is non-pathogenic to humans and it is incapable of producing certain endotoxins. Second, it has a long history of safe use following centuries of commercial exploitation for various purposes. This has led to wide public acceptability. Third, the extensive commercial use and research devoted to the organism has resulted in a wealth
- 30 of knowledge about the genetics and physiology as well as large-scale fermentation characteristics of *Saccharomyces cerevisiae*.

A review of the principles of heterologous gene expression in *Saccharomyces cerevisiae* and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a

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- 5 vehicle for the expression of heterologous genes", Yeasts, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

Several types of yeast vectors are available, including integrative vectors, which require recombination with the host genome for their maintenance, and autonomously replicating
10 plasmid vectors.

In order to prepare the transgenic *Saccharomyces*, expression constructs are prepared by inserting the nucleotide sequence of the present invention into a construct designed for expression in yeast. Several types of constructs used for heterologous expression have
15 been developed. The constructs contain a promoter active in yeast fused to the nucleotide sequence of the present invention, usually a promoter of yeast origin, such as the GAL1 promoter, is used. Usually a signal sequence of yeast origin, such as the sequence encoding the SUC2 signal peptide, is used. A terminator active in yeast ends the expression system.

20 For the transformation of yeast several transformation protocols have been developed. For example, a transgenic *Saccharomyces* according to the present invention can be prepared by following the teachings of Hinnen et al (1978, Proceedings of the National Academy of Sciences of the USA 75, 1929); Beggs, J D (1978, Nature, London, 275,
25 104); and Ito, H et al (1983, J Bacteriology 153, 163-168).

The transformed yeast cells are selected using various selective markers. Among the markers used for transformation are a number of auxotrophic markers such as LEU2, HIS4 and TRP1, and dominant antibiotic resistance markers such as aminoglycoside
30 antibiotic markers, e.g. G418.

Another host organism is a plant. The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material.

5

A transgenic plant of the invention may be produced from any plant such as the seed-bearing plants (angiosperms), and conifers. Angiosperms include dicotyledons and monocotyledons. Examples of dicotyledonous plants include tobacco, (*Nicotiana plumbaginifolia* and *Nicotiana tabacum*), arabidopsis (*Arabidopsis thaliana*), *Brassica napus*, *Brassica nigra*, *Datura innoxia*, *Vicia narbonensis*, *Vicia faba*, pea (*Pisum sativum*), cauliflower, carnation and lentil (*Lens culinaris*). Examples of monocotyledonous plants include cereals such as wheat, barley, oats and maize.

Techniques for producing transgenic plants are well known in the art. Typically, either whole plants, cells or protoplasts may be transformed with a suitable nucleic acid construct encoding a zinc finger molecule or target DNA (see above for examples of nucleic acid constructs). There are many methods for introducing transforming DNA constructs into cells, but not all are suitable for delivering DNA to plant cells. Suitable methods include *Agrobacterium* infection (see, among others, Turpen *et al.*, 1993, J. Virol. Methods, 42: 227-239) or direct delivery of DNA such as, for example, by PEG-mediated transformation, by electroporation or by acceleration of DNA coated particles. Acceleration methods are generally preferred and include, for example, microprojectile bombardment. A typical protocol for producing transgenic plants (in particular monocotyledons), taken from U.S. Patent No. 5, 874, 265, is described below.

An example of a method for delivering transforming DNA segments to plant cells is microprojectile bombardment. In this method, non-biological particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like.

A particular advantage of microprojectile bombardment, in addition to it being an effective means of reproducibly stably transforming both dicotyledons and monocotyledons, is that neither the isolation of protoplasts nor the susceptibility to *Agrobacterium* infection is required. An illustrative embodiment of a method for

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- 5 delivering DNA into plant cells by acceleration is a Biolistics Particle Delivery System, which can be used to propel particles coated with DNA through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with plant cells cultured in suspension. The screen disperses the tungsten-DNA particles so that they are not delivered to the recipient cells in large aggregates. It is believed that without a
- 10 screen intervening between the projectile apparatus and the cells to be bombarded, the projectiles aggregate and may be too large for attaining a high frequency of transformation. This may be due to damage inflicted on the recipient cells by projectiles that are too large.
- 15 For the bombardment, cells in suspension are preferably concentrated on filters. Filters containing the cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate. If desired, one or more screens are also positioned between the gun and the cells to be bombarded. Through the use of techniques set forth herein one may obtain up to 1000 or more clusters of cells transiently expressing
- 20 a marker gene ("foci") on the bombarded filter. The number of cells in a focus which express the exogenous gene product 48 hours post-bombardment often range from 1 to 10 and average 2 to 3.

- After effecting delivery of exogenous DNA to recipient cells by any of the methods
- 25 discussed above, a preferred step is to identify the transformed cells for further culturing and plant regeneration. This step may include assaying cultures directly for a screenable trait or by exposing the bombarded cultures to a selective agent or agents.

- An example of a screenable marker trait is the red pigment produced under the control
- 30 of the R-locus in maize. This pigment may be detected by culturing cells on a solid support containing nutrient media capable of supporting growth at this stage, incubating the cells at, e.g., 18°C and greater than $180 \mu\text{E m}^{-2} \text{s}^{-1}$, and selecting cells from colonies (visible aggregates of cells) that are pigmented. These cells may be cultured further, either in suspension or on solid media.

35

5 An exemplary embodiment of methods for identifying transformed cells involves exposing the bombarded cultures to a selective agent, such as a metabolic inhibitor, an antibiotic, herbicide or the like. Cells which have been transformed and have stably integrated a marker gene conferring resistance to the selective agent used, will grow and divide in culture. Sensitive cells will not be amenable to further culturing.

10

To use the bar-bialaphos selective system, bombarded cells on filters are resuspended in nonselective liquid medium, cultured (e.g. for one to two weeks) and transferred to filters overlaying solid medium containing from 1-3 mg/l bialaphos. While ranges of 1-3 mg/l will typically be preferred, it is proposed that ranges of 0.1-50 mg/l will find utility in the practice of the invention. The type of filter for use in bombardment is not believed to be particularly crucial, and can comprise any solid, porous, inert support.

Cells that survive the exposure to the selective agent may be cultured in media that supports regeneration of plants. Tissue is maintained on a basic media with hormones for about 2-4 weeks, then transferred to media with no hormones. After 2-4 weeks, shoot development will signal the time to transfer to another media.

Regeneration typically requires a progression of media whose composition has been modified to provide the appropriate nutrients and hormonal signals during sequential developmental stages from the transformed callus to the more mature plant. Developing plantlets are transferred to soil, and hardened, e.g., in an environmentally controlled chamber at about 85% relative humidity, 600 ppm CO₂, and 250 $\mu\text{E m}^{-2} \text{s}^{-1}$ of light. Plants are preferably matured either in a growth chamber or greenhouse. Regeneration will typically take about 3-12 weeks. During regeneration, cells are grown on solid media in tissue culture vessels. An illustrative embodiment of such a vessel is a petri dish. Regenerating plants are preferably grown at about 19°C to 28°C. After the regenerating plants have reached the stage of shoot and root development, they may be transferred to a greenhouse for further growth and testing.

- 5 Genomic DNA may be isolated from callus cell lines and plants to determine the presence of the exogenous gene through the use of techniques well known to those skilled in the art such as PCR and/or Southern blotting.

Several techniques exist for inserting the genetic information, the two main principles
10 being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

- 15 Thus, in one aspect, the present invention relates to a vector system which carries a construct encoding a variant xylanase polypeptide according to the present invention and which is capable of introducing the construct into the genome of a plant.

The vector system may comprise one vector, but it can comprise at least two vectors.
20 In the case of two vectors, the vector system is normally referred to as a binary vector system. Binary vector systems are described in further detail in Gynheung An et al. (1980), Binary Vectors, *Plant Molecular Biology Manual A3*, 1-19.

One extensively employed system for transformation of plant cells with a given
25 promoter or nucleotide sequence or construct is based on the use of a Ti plasmid from *Agrobacterium tumefaciens* or a Ri plasmid from *Agrobacterium rhizogenes* (An et al. (1986), *Plant Physiol.* 81, 301-305 and Butcher D.N. et al. (1980), *Tissue Culture Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208).

- 30 Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above.

5 B. Uses

In a general sense, a variant xylanase of the invention may be used to alter, for example reduce, the viscosity derived from the presence of hemicellulose or arabinoxylan in a solution or system comprising plant cell wall material. Typically said plant cell wall
10 materials will comprise one or more xylanase inhibitors. Suitably said plant cell wall materials will be processed at a preferred temperature.

Specifically, a variant xylanase of the invention may be used in processing plant materials for use as foodstuffs, such as animal feed, in processing cereals, in starch
15 production, in baking and in the processing of wood pulp to make paper.

The variant xylanase of the present invention may be used in the preparation of a dough and baked products that are obtained by baking such a dough, and in the preparation of noodle and pasta products.

20

The variant xylanase of the present invention may be used in a dough improving composition comprising.

Preparation of foodstuffs

25

A variant xylanase of the invention may be used to process plant materials such as cereals that are used in foodstuffs including animal feed. As used herein, the term "cereal" means any kind of grain used for food and/or any grass producing this grain such as but not limited to any one of wheat, milled wheat, barley, maize, sorghum, rye,
30 oats, triticale and rice or combinations thereof. In one preferred embodiment, the cereal is a wheat cereal.

The xylan in the food and/or feed supplement is modified by contacting the xylan with the variant xylanase of the present invention.

35

- 5 As used herein, the term "contacting" includes but is not limited to spraying, coating, impregnating or layering the food and/or feed supplement with the variant xylanase enzyme of the present invention.

10 In one embodiment, the food and/or feed supplement of the present invention may be prepared by mixing the variant xylanase enzyme directly with a food and/or feed supplement. By way of example, the variant xylanase enzyme may be contacted (for example, by spraying) onto a cereal-based food and/or feed supplement such as milled wheat, maize or soya flour.

- 15 It is also possible to incorporate the variant xylanase enzyme into a second (and different) food and/or feed or drinking water which is then added to the food and/or feed supplement of the present invention. Accordingly, it is not essential that the variant xylanase enzyme provided by the present invention is incorporated into the cereal-based food and/or feed supplement itself, although such incorporation forms a
20 particularly preferred aspect of the present invention.

In one embodiment of the present invention, the food and/or feed supplement may be combined with other food and/or feed components to produce a cereal-based food and/or feed. Such other food and/or feed components may include one or more other
25 (preferably thermostable) enzyme supplements, vitamin food and/or feed supplements, mineral food and/or feed supplements and amino acid food and/or feed supplements. The resulting (combined) food and/or feed supplement comprising possibly several different types of compounds can then be mixed in an appropriate amount with the other food and/or feed components such as cereal and protein supplements to form a
30 human food and/or an animal feed.

In one preferred embodiment, the food and/or feed supplement of the present invention can be prepared by mixing different enzymes having the appropriate activities to produce an enzyme mix. By way of example, a cereal-based food and/or feed
35 supplement formed from e.g. milled wheat or maize may be contacted (e.g. by

5 spraying) either simultaneously or sequentially with the xylanase enzyme and other enzymes having appropriate activities. These enzymes may include but are not limited to any one or more of an amylase, a glucoamylase, a mannanase, an a galactosidase, a phytase, a lipase, a galactolipase, a phospholipase, a glucanase, an arabinofuranosidase, a pectinase, a protease, a glucose oxidase, a hexose oxidase and a xylanase. Enzymes
10 having the desired activities may for instance be mixed with the xylanase of the present invention either before contacting these enzymes with a cereal-based food and/or feed supplement or alternatively such enzymes may be contacted simultaneously or sequentially on such a cereal based supplement. The food and/or feed supplement is then in turn mixed with a cereal-based food and/or feed to prepare the final food and/or
15 feed. It is also possible to formulate the food and/or feed supplement as a solution of the individual enzyme activities and then mix this solution with a food and/or feed material prior to processing the food and/or feed supplement into pellets or as a mash.

The variant xylanase of the present invention may be used in combination with other
20 components. Thus, the present invention also relates to combinations.

The combination of the present invention comprises the variant of the present invention and another component which is suitable for animal or human consumption.

25 Further examples of other suitable components include one or more of: thickeners, gelling agents, emulsifiers, binders, crystal modifiers, sweeteners (including artificial sweeteners), rheology modifiers, stabilisers, anti-oxidants, dyes, enzymes, carriers, vehicles, excipients, diluents, lubricating agents, flavouring agents, colouring matter, suspending agents, disintegrants, granulation binders etc. These other components
30 may be natural. These other components may be prepared by use of chemical and/or enzymatic techniques.

As used herein the term "component suitable for animal or human consumption" means a compound which is or can be added to the composition of the present

- 5 invention as a supplement which may be of nutritional benefit, a fibre substitute or have a generally beneficial effect to the consumer.

Bakery products

- 10 The present invention provides the use of a variant xylanase polypeptide of the invention in a process for preparing a foodstuff. Typical bakery (baked) products in accordance with the present invention include bread - such as loaves, rolls, buns, pizza bases etc. - pretzels, tortillas, cakes, cookies, biscuits, crackers etc. The preparation of foodstuffs such as bakery products is well known in the art.

15

- The present invention advantageously provides a variant xylanase polypeptide capable of improving the properties of flour based doughs and products made from such doughs. This is, in respect of baked products, achieved by providing a method for preparing baked products which have highly desirable characteristics with respect to bread volume, crumb structure and appearance and which additionally have an extended shelf life as reflected in an enhanced softness, i.e. the staling of the baked products is retarded relative to a baked product made without use of the enzyme of the invention. Although it is presently preferred to use the method for the manufacturing of yeast leavened bread products such as bread loaves, rolls or toast bread, the use of the method for any other types of doughs and dough based products such as noodle and pasta products and cakes, the quality of which can be improved by the addition of the enzymes of the invention, is also contemplated.
- 20
- 25

- The present invention further relates to pasta doughs, noodle doughs and cake doughs or batters comprising the variant xylanase polypeptide and finished products made from such doughs or batters.
- 30

5

Starch production

A variant xylanase of the invention may also be used in starch production from plant materials derived from cereals and tubers, such as potatoes.

Processing of wood pulp

A variant xylanase of the invention may also be used in processing wood pulp, for example in the preparation of paper.

The present invention will now be further described with reference to the following examples which are intended to be illustrative only and non-limiting.

20 **EXAMPLES****Example 1 - Purification and characterisation of wheat endogenous xylanase inhibitor.**

25 2 kg wheat flour (Danish reform, batch 99056) was extracted with water, using a flour:water ratio of 1:2, during 10 minutes of stirring. The soluble endogenous xylanase inhibitor was separated from the flour-water slurry by centrifugation. The extraction and centrifugation was performed at 4°C. The inhibitor was purified from the water extract by the following chromatographic techniques and concentration techniques: HPLC-SEC, HPLC-CIEC, rotary evaporation, HPLC-HIC, HPLC-SEC and rotary evaporation. The xylanase inhibitor could be monitored and quantified during purification, using the following quantification method.

5

Inhibitor quantification method

1 XIU (Xylanase Inhibitor Unit) is defined as the amount of inhibitor that decreases 1 TXU to 0.5 TXU under the conditions described below.

10

The xylanase used in this assay is *Bacillus subtilis* wild type xylanase.

250 µl xylanase solution containing 12 TXU/ml, approx. 100 µl xylanase inhibitor solution and citric acid (0.1 M) - di-sodium-hydrogen phosphate (0.2 M) buffer, pH 5, to react a reaction volume of 1000 µl is pre-incubated for 5 minutes at 40°C. At t = 5 minutes, 1 Xylazyme (Megazyme, Ireland) tablet is added to the reaction mixture. At t = 15 minutes the reaction is terminated, by addition of 10 ml 2 % TRIS/NaOH, pH 12. The solution is filtered and the absorbency of the supernatant is measured at 590 nm. By choosing several different concentrations of inhibitor in the above assay, it is possible to create a plot of OD versus inhibitor concentration. Using the slope (a) and intercept (b) from this plot and the concentration of the xylanase it is possible to calculate the amount of XIU in a given inhibitor solution (equation 1).

Equation 1 amount of XIU in solution = ((b/2)/-a)/ TXU

25

From the endogenous xylanase inhibitor purification, the following inhibitor yield was recovered (table 1).

Table 1. Wheat endogenous xylanase inhibitor recovery after purification.

30

Sample	Amount	XIU	XIU, total	Recovery, %
Flour	2000 g	590/g	1.180.000	100
Purified inhibitor	90 ml	4658/ml	419.220	35.5

The inhibitor sample was pure and free from wheat endogenous xylanolytic activities.

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5

Example 2 - Site-directed mutagenesis on xylanases.

Specific mutants of *Bacillus subtilis* xylanase may be obtained by site directed mutagenesis of the wild type enzyme, by the use of any of a number of commercially available mutagenesis kits. An example of how to obtain the D11F mutant using the Quick Exchange kit, available from Stratagene Cloning Systems, 11011 North Torrey Pines Road, La Jolla, CA 92037, USA is given below :

The DNA sequence encoding *Bacillus subtilis* xylanase A has been published by Paice et al., 1986.

The sequence of the coding region is as follows, with the sequence encoding the mature part of the protein shown in capitals:

```

20  catatgtttaagtttaaaaaagaatttcttagtgattatcggcagctttaatgagtatt
    agcttgttttcggcaaccgcctctgcagCTAGCACAGACTACTGGCAAAATGGACTGAT
    GGGGGCGGTATAGTAAACGCTGTCAATGGGTCTGGCGGGAATTACAGTGTTAATTGGTCT
    AATACCGGAAATTTTGTGTGGTAAAGTTGGACTACAGGTCGCCATTTAGGACGATA
    AACTATAATGCCGGAGTTTGGGCGCCGAATGGCAATGGATATTTAACTTTATATGGTTGG
25  ACGAGATCACCCTCTCATAGAAATATATGTAGTGGATTTCATGGGTACTTATAGACCTACT
    GGAACGTATAAAGGTACTGTAAAAAGTGATGGGGGTACATATGACATATATACAACATACA
    CGTTATAACGCACCTTCCATTGATGGCGATCGCACTACTTTACGCAGTACTGGAGTGT
    CGCCAGTCGAAGAGACCAACCGGAAGCAACGCTACAATCACTTTCAGCAATCATGTGAAC
    GCATGGAAGAGCCATGGAATGAATCTGGGCAGTAATTGGGCTTACCAAGTCATGGCGACA
30  GAAGGATATCAAGTAGTGAAGTTCTAACGTAACAGTGGTAA

```

The part of the gene encoding the mature part of the wild type enzyme may be expressed intracellularly in *E.coli* by methods well known to people skilled in the art of molecular biology. For example :

35

1. Generating a copy of the capitalised part of the above described gene by use of the Polymerase Chain Reaction (PCR) with an added NdeI restriction enzyme site

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- 5 (CATATG) before the GCTAGCACA and an added HindIII restriction site (AAGCTT) after the GTGTGGTAA.
2. Inserting the resultant modified copy of the gene by use of the above mentioned enzymes into the expression vector pET24a(+), which can be obtained from Novagen, Inc. 601 Science Drive, Madison, WI 53711, USA.
- 10 3. Transforming into a suitable *E.coli* strain and expression by fermentation as described by the vendor of pET24a(+).

Our D11F mutant enzyme may be obtained by using the "Quick Exchange" mutagenesis kit according to the manufacturer, and using the above described *Bacillus*
15 *subtilis* wild type xylanase-pET24a(+) construct and the following PCR mutagenesis primers :

Sense primer:

CTACTGGCAAAATTGGACTTTTGGAGGAGGTATAGTAAACGCTG

20

Antisense primer :

CAGCGTTTACTATACCTCCTCCAAAAGTCCAATTTGCCAGTAG

The mutant enzyme is expressed and purified using the same protocols as for the wild
25 type enzyme.

Example 3 - Inhibition studies of xylanase mutants.

Xylanase mutants expressed in *E. coli* (see Example 2) were fermented and purified
30 (meaning no other xylanolytic activity was present in the purified preparation) using a de-salting step and a cation exchange chromatography step.

These pure xylanase mutant preparations were diluted to 12 TXU/ml using 0.1 M citric acid - 0.2 M di-sodium-hydrogen phosphate, pH 5.0 and used in the following assay.

35

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- 5 A stable inhibitor preparation was made according to the protocol described in Example 1. This stable inhibitor preparation is used as stock for all xylanase-xylanase inhibitor studies. Using the inhibitor quantification method described in Example 1, the inhibitor preparation was analysed to contain 126 XIU/ml.

10 Assay

- To 250 μ l diluted xylanase mutant preparations, are added 0, 10, 25, 50 or 100 μ l inhibitor preparation, respectively. To these inhibitor-xylanase mixtures were added 0.1 M citric acid - 0.2 M *di*-sodium-hydrogen phosphate, pH 5.0 making the end-
 15 volume 1000 μ l. These reaction mixtures were pre-incubated for 5 minutes at 40°C. Hereafter 1 Xylazyme tablet (Megazyme, Ireland) were added to all inhibitor-xylanase mixtures. After 10 minutes of incubation at 40°C, the reactions were terminated, by adding 10 ml 2% Tris/NaOH, pH 12.0. The mixtures were centrifuged and the liberated blue colour from the substrate was measured at 590 nm.

20

The results in respect of one variant xylanase are presented in table 2.

Table 2. Relative inhibition of a xylanase mutant and parent xylanase (here wildtype enzyme) as a function of xylanase inhibitor.

25

Xylanase	Xylanase Inhibitor (XIU/ml)				
	0	1,26	3,15	6,3	12,6
	Relative inhibition, %				
Wildtype	100	77	48	29	23
Mutant ID : D121F/N8Y/S2 2P/N29T/T44V /F48N/T50V/V 57I/A59E/N61S /S74N/P75A/P9 0A/T93N	100	101	88	70	50

From the results in Table 2, it can be seen that the variant xylanase has a changed sensitivity towards the wheat endogenous xylanase inhibitor.

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- 5 Other suitable variant xylanases having changed inhibitor sensitivity include those based on *B. subtilis* xylanase with the following mutations:
- i) D11F/K99N/T111M/T126Q/Y128F/A142V/T143S/T147S/K154R/H156K/N163S/M169L/S179R
 - ii) N32K/K99N/T111M/T126Q/Y128F/A142V/T143S/T147S/K154R/H156K/N163S/M169L/S179R
 - 10 iii) T123D/N8Y/S22P/N29T/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/P90A/T93N

Example 4 - Thermostability studies of xylanase mutants.

- 15 Xylanase mutants expressed in *E. coli* (see Example 2) were fermented and purified (meaning no other xylanolytic activity were present in the purified preparation).

- The xylanase mutants were analysed according to the "thermostability assay" mentioned above.

- Suitable variant xylanases having altered thermostability include those based on *B. subtilis* xylanase with the following mutations:
- i) D11F/K99N/T111M/T126Q/Y128F/A142V/T143S/T147S/K154R/H156K/N163S/M169L/S179R
 - 25 ii) N32K/K99N/T111M/T126Q/Y128F/A142V/T143S/T147S/K154R/H156K/N163S/M169L/S179R
 - iii) T123D/N8Y/S22P/N29T/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/P90A/T93N
 - 30 iv) D121F/N8Y/S22P/N29T/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/P90A/T93N

Variant xylanases had an altered thermostability compared to the wild type xylanase.

35

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5 **Example 5. Temperature optimum studies of xylanase mutants.**

Xylanase mutants expressed in *E. coli* (see Example 2) were fermented and purified (meaning no other xylanolytic activity were present in the purified preparation).

- 10 The xylanase mutants were analysed according to the "temperature optimum assay" mentioned above.

Suitable variant xylanases having altered thermostability include those based on *B. subtilis* xylanase with the following mutations:

- 15 i) D11F/K99N/T111M/T126Q/Y128F/A142V/T143S/T147S/K154R/H156K/N163S/M169L/S179R
 ii) N32K/K99N/T111M/T126Q/Y128F/A142V/T143S/T147S/K154R/H156K/N163S/M169L/S179R
 iii) T123D/N8Y/S22P/N29T/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/P90A/
 20 T93N
 iv) D121F/N8Y/S22P/N29T/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/P90A/T93N

- 25 Variant xylanases have changed temperature optima compared to the wild type xylanase.

Example 6. Xylanase variant having changed inhibitor-sensitivity and changed thermo-sensitivity – (A)

- 30 A variant of *Bacillus subtilis* 168 xylanase A, with changed inhibitor sensitivity and with altered thermo-sensitivity may be engineered by combining mutation D11F (changing inhibitor sensitivity) with mutations K99N, T111M, T126Q, Y128F, A142V, T143S, T147S, K154R, H156K, N163S, M169L, S179R (changing thermo-sensitivity).

35

5 **Example 7. Xylanase variant having changed inhibitor-sensitivity and changed thermo-sensitivity – (B)**

A variant of *Bacillus subtilis* 168 xylanase A, with changed inhibitor sensitivity and with altered thermo-sensitivity may be engineered by combining mutation N32K
10 (changing inhibitor sensitivity) with mutations K99N, T111M, T126Q, Y128F, A142V, T143S, T147S, K154R, H156K, N163S, M169L, S179R (changing thermo-sensitivity).

15 **Example 8. Xylanase variant having changed inhibitor-sensitivity and changed thermo-sensitivity – (C).**

A variant of *Bacillus subtilis* 168 xylanase A, with changed inhibitor sensitivity and with altered thermo sensitivity may be engineered by combining mutation T123D (changing inhibitor sensitivity) with mutations N8Y, S22P, N29T, T44V, F48N, T50V,
20 V57I, A59E, N61S, S74N, P75A, P90A, T93N (changing thermo-sensitivity).

Example 9. Xylanase variant having changed inhibitor-sensitivity and changed thermo-sensitivity – (D).

25 A variant of *Bacillus subtilis* 168 xylanase A, with changed inhibitor sensitivity and with altered thermo sensitivity may be engineered by combining mutation D121F (changing inhibitor sensitivity) with mutations N8Y, S22P, N29T, T44V, F48N, T50V, V57I, A59E, N61S, S74N, P75A, P90A, T93N (changing thermo-sensitivity).

30 **Example 10. Inhibition studies of xylanase variants**

B. subtilis xylanase mutants were obtained by site directed mutagenesis of the wild type enzyme (see example 2). The following variants were used in this example:

35 *Mut 1:*

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5 S2A/N8Y/I15M/S22P/N29T/S31Q/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/
D121F

Mut 2:

S2A/N8Y/I15M/S22P/N29T/S31Q/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/
T123D

10

These mutants were expressed in *E. coli* (see Example 2), fermented and purified (meaning no other xylanolytic activity was present in the purified preparation) using a de-salting step and a cation exchange chromatography step.

15 These pure xylanase mutant preparations were evaluated for inhibitor sensitivity, using the "Xylanase inhibitor assay" mentioned previously.

A stable inhibitor preparation was made according to the protocol described in Example 1. This stable inhibitor preparation was used as stock for all xylanase-
20 xylanase inhibitor studies. Using the "inhibitor quantification method" described in example 1, the inhibitor preparation was analysed to contain 126 XIU/ml.

The results are presented in Table 3.

25 Table 3. Relative inhibition of xylanase mutants and a parent xylanase (here wild type *B. subtilis* enzyme) as a function of xylanase inhibitor concentration, XIU/ml. The activity is expressed as a percentage of the activity at a given inhibitor concentration relative to the activity when XIU/ml =0.

	Inhibitor concentration, XIU/ml				
Xylanase	0	1,26	3,15	6,3	12,6
Wt	100	76	50	30	23
Mut 1	100	92	73	51	33

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- 5 As can be seen from Table 3, the mutations introduced in the parent xylanase changed the sensitivity of the variant xylanases towards the wheat endogenous xylanase inhibitor.

In addition, the xylanase mutant (Mut 2) has was less stable to the wheat endogenous
10 xylanase inhibitor at the concentration 1,26 XIU/ml.

Example 11. Thermostability studies of xylanase mutants

- B. subtilis* xylanase mutants were obtained by site directed mutagenesis of the wild
15 type enzyme (see example 2). The following variants were used in this example:

Mut 1:

S2A/N8Y/I15M/S22P/N29T/S31Q/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/
D121F

- 20 *Mut 2:*

S2A/N8Y/I15M/S22P/N29T/S31Q/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/
T123D

- These mutants were expressed in *E. coli* (see Example 2), fermented and purified
25 (meaning no other xylanolytic activity was present in the purified preparation) using a de-salting step and a cation exchange chromatography step.

These xylanase mutant preparations were evaluated for thermostability, using the
"thermostability assay" mentioned previously.

30

The results are presented in Tables 4 -7.

Table 4. Thermostability of a parent xylanase (here wild type *B. subtilis* xylanase) and variants thereof as a function of incubation time at 50°C. The results are represented

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- 5 as a percentage of the activity at given incubation time relative to the activity when time = 0 hour.

Time (hr)	wt	Mut 1	Mut 2
0	100	100	100
0.5	89	106	99
1	91	101	102
2	88	105	100
3	86	103	100
4	87	104	97

- 10 Table 5. Thermostability of parent xylanase (here wild type *B. subtilis* xylanase) and variants thereof as a function of incubation time at 55°C. The results are represented as a percentage of the activity at given incubation time relative to the activity when time = 0 hour.

Time (hr)	wt	Mut 1	Mut 2
0	100	100	100
0.5	60	105	99
1	40	102	92
2	30	101	95
3	23	102	88
4	18	99	86

15

- Table 6. Thermostability of a parent xylanase (here wild type *B. subtilis* xylanase) and variants thereof as a function of incubation time at 60°C. The results are represented as a percentage of the activity at given incubation time relative to the activity when time = 0 hour.

20

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Time (hr)	wt	Mut 1	Mut 2
0	100	100	100
0.5	-	92	101
1	-	75	85
2	-	56	68
3	-	44	50
4	-	33	39

5

Table 7. Thermostability of a parent xylanase (here wild type *B. subtilis* xylanase) and variants thereof as a function of incubation time at 65°C. The results are represented as a percentage of the activity at given incubation time relative to the activity when time = 0 hour.

10

Time (hr)	wt	Mut 1	Mut 2
0	100	100	100
0.5	-	2	4
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-

15

As can be seen from Tables 4–7, the mutations introduced in the parent xylanase changed the sensitivity of the variant xylanases to temperature (thermostability). At approx. 60°C two of the variants are more stable than the parent xylanase (here wild type).

Example 12. Temperature optimum studies of xylanase mutants.

B. subtilis xylanase mutants were obtained by site directed mutagenesis of the wild type enzyme (see example 2). The following variants were used in this example:

20

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5 *Mut 1:*

S2A/N8Y/I15M/S22P/N29T/S31Q/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/
D121F

Mut 2:

S2A/N8Y/I15M/S22P/N29T/S31Q/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/
10 T123D

These mutants were expressed in *E. coli* (see Example 2), fermented and purified (meaning no other xylanolytic activity was present in the purified preparation) using a de-salting step and a cation exchange chromatography step.

15

These pure xylanase mutant preparations were evaluated for temperature optimum, using the "temperature optimum assay" mentioned previously.

The results are presented in Table 8.

20

Table 8. Temperature optimum of a parent xylanase (here wild type *B. subtilis* xylanase) and variants thereof. The results are represented as a percentage of the activity at a given temperature relative to the highest activity.

Temp (°C)	wt	Mut 1	Mut 2
30	37	22	27
40	66	46	51
50	100	86	87
60	17	100	100

25

As can be seen from Table 8, the mutations introduced in the parent xylanase changed the temperature optimum profile of the variant xylanases compared to the parent xylanase (here wild type *B. subtilis*). The variants have an approx. 10°C higher optimum than the parent xylanase.

30

5 SUMMARY

In summary, the present invention provides a means for altering the inhibitor sensitivity of a xylanase enzyme combined with a means for changing the thermostability and/or the temperature optimum of the enzyme. In this way, the inhibitor sensitivity and the thermosensitivity of the xylanase enzyme can be independently controlled.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing
10 from the scope and spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or
15 related fields are intended to be within the scope of the following claims.

5 References

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- Rouau, X. and Surget, A. (1998). Evidence for the presence of a pentosanase inhibitor in wheat flour. Journal of Cereal Science. 28, 63-70.
- 30 Sibbesen, O. and Soerensen, J.F. (1999). Bacterial xylanase. UK A 9828599.2.

SEQUENCE LISTINGS

The amino acid sequence of the mature *Bacillus subtilis* xylanase (SEQ ID. No. 1).

```

1      10 11      20 21      30 31      40 41      50 51      60
ASTDYWQNW T DGGGIVNAVN GSGGNYSVNW SNTGNFVVGK GWTTGSPFRT INYNAGVWAP

61      70 71      80 81      90 91      100 101      110 111      120
NGNGYLTLYG WTRSPLEIYY VVDSWGTYRP TGTYKGT VKS DGGTYDIYTT TRYNA PSIDG

121     130 131     140 141     150 151     160 161     170 171     180
DRTTFTQYWS VRQSKRPTGS NATITFSNHV NAWKSHGMNL GSNWAYQVMA TEGYQSSGSS

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NVTVW

```

Amino acid sequences derived from wheat flour xylanase inhibitor

A chain of inhibitor

N-terminal:

GAPVARAVEAVAPFGVCYDTKTLGNNLGGYAVPNV (35aa) SEQ ID NO. 2

C-terminal:

KRLGFSRLPHFTGCGGL (17aa) SEQ ID NO. 3

B chain of inhibitor

N-terminal:

LPVPAPVTKDPATSLYTIPFH (21aa) SEQ ID NO. 4

Lys-C digested B Chain:

LLASLPRGSTGVAGLANGLALPAQVASAQK (31aa) SEQ ID NO. 5

GGSPAHIYISARFIEVGDTRVPSVE (24aa) SEQ ID NO. 6

VNVGVLAACAPSK (13aa) SEQ ID NO. 7

VANRFLCLPTGGPGVAIFGGGPVPWPQFTQSMPYTLVVVK SEQ ID NO. 8

CLAIMS

1. A method of altering the sensitivity of a xylanase polypeptide to an inhibitor and of altering the thermo-sensitivity of said xylanase polypeptide, which method comprises modifying a parent xylanase polypeptide to provide a variant xylanase polypeptide, and testing the sensitivity of said variant xylanase polypeptide to a xylanase inhibitor compared with the parent xylanase enzyme and testing the thermosensitivity of said variant xylanase polypeptide compared with the parent xylanase enzyme and selecting a variant xylanase polypeptide having an altered sensitivity to a xylanase inhibitor and an altered thermo-sensitivity compared with the parent xylanase enzyme.
2. A method according to claim 1 wherein said variant polypeptide is derived from a family 11 xylanase.
3. A method according to claim 1 wherein there are at least two of said amino acid modifications.
- 5 4. A method according to any one of the preceding claims wherein said amino acid modification is at any one or more of amino acid residues:

2, 8, 11, 12, 13, 15, 17, 22, 29, 31, 32, 34, 44, 48, 50, 57, 59, 61, 74, 75, 90, 93, 99, 111, 113, 114, 119, 120, 121, 122, 123, 124, 126, 128, 142, 143, 147, 154, 156, 163,
10 169, 175 and 179, or combinations hereof, based on the amino acid numbering of *B. subtilis* xylanase shown as SEQ ID No. 1, or the equivalent residues in other homologous xylanase polypeptides.
5. A method according to any one of the preceding claims wherein the inhibitor is an inhibitor found naturally in plant tissues.

6. A method according to any one of the preceding claims wherein the sensitivity to an inhibitor is reduced.
7. A variant xylanase polypeptide obtainable by the method according to any one of claims 1 to 6.
8. A variant xylanase polypeptide, or fragment thereof having xylanase activity, comprising one or more amino acid modifications such that the polypeptide or fragment thereof has an altered sensitivity to a xylanase inhibitor as compared with a parent xylanase enzyme and has an altered thermo-sensitivity as compared with a parent xylanase enzyme.
9. A variant polypeptide according to claim 8 which is derived from a family 11 xylanase.
10. A variant xylanase polypeptide, or fragment thereof having xylanase activity, according to any one of claims 8-9 wherein there are at least two of said amino acid modifications.
11. A variant xylanase polypeptide, or fragment thereof having xylanase activity according to any one of claims 8-10 wherein said amino acid modification is at any one or more of amino acid residues:
2, 8, 11, 12, 13, 15, 17, 22, 29, 31, 32, 34, 44, 48, 50, 57, 59, 61, 74, 75, 90, 93, 99,
111, 113, 114, 119, 120, 121, 122, 123, 124, 126, 128, 142, 143, 147, 154, 156, 163,
169, 175 and 179, or combinations thereof, based on the amino acid numbering of *B. subtilis* xylanase shown as SEQ ID No. 1, or the equivalent residues in other homologous xylanase polypeptides.
12. A variant xylanase polypeptide according to any one of claims 8-11 wherein the inhibitor is an inhibitor found naturally in plant tissues.

13. A variant xylanase polypeptide according to any one of claims 8-12 wherein the sensitivity to an inhibitor is reduced.
14. A method of altering the sensitivity of a xylanase polypeptide to an inhibitor and of altering the thermo-sensitivity of said xylanase polypeptide enzyme, which method comprises modifying one or more amino acid residues of said enzyme such that the polypeptide or a fragment thereof has an altered sensitivity to a xylanase inhibitor and has an altered thermo-sensitivity as compared with the parent xylanase enzyme.
15. A method according to claim 14 wherein said variant polypeptide is that defined in any one of claims 8 to 13.
16. A method according to claim 14 or claim 15 wherein the sensitivity of the enzyme to an inhibitor is reduced.
17. A composition comprising a variant polypeptide according to any one of claims 7 to 13.
18. A method of degrading or modifying a plant cell wall which method comprises contacting said plant cell wall with a polypeptide according to any one of claims 7 to 13 or a composition according to claim 17.
19. A method of processing a plant material which method comprises contacting said plant material with a polypeptide according to any one of claims 7 to 13 or composition according to claim 17.
20. A nucleotide sequence encoding a variant polypeptide according to any one of claims 7 to 13.

- 5 21. A construct comprising the nucleotide sequence according to claim 20.
22. Use of a variant polypeptide according to any one of claims 7 to 13 in a method of modifying plant materials.
23. Use of a variant polypeptide according to any one of claims 7 to 13 in any one or more of: baking, processing cereals, starch production, in processing wood, enhancing the bleaching of wood pulp.

Figure 1 continued

B. subtilis xylA	105	120	121	133	136	150	151	165	166	180
B. subtilis xylB	106	122	123	134	137	152	153	167	168	181
A. baumannii xylA	107	124	125	136	139	154	155	169	170	182
A. baumannii xylB	108	126	127	138	141	156	157	171	172	183
A. caviae xylA	109	128	129	140	143	158	159	173	174	184
A. caviae xylB	110	130	131	142	145	160	161	175	176	185
C. carboxum xylA	111	132	133	144	147	162	163	177	178	186
C. carboxum xylB	112	134	135	146	149	164	165	179	180	187
C. carboxum xylC	113	136	137	148	151	166	167	181	182	188
C. carboxum xylD	114	138	139	150	153	168	169	183	184	189
C. carboxum xylE	115	140	141	152	155	170	171	185	186	190
C. carboxum xylF	116	142	143	154	157	172	173	187	188	191
C. carboxum xylG	117	144	145	156	159	174	175	189	190	192
C. carboxum xylH	118	146	147	158	161	176	177	191	192	193
C. carboxum xylI	119	148	149	160	163	178	179	193	194	194
C. carboxum xylJ	120	150	151	162	165	180	181	195	196	195
C. carboxum xylK	121	152	153	164	167	182	183	197	198	196
C. carboxum xylL	122	154	155	166	169	184	185	199	200	197
C. carboxum xylM	123	156	157	168	171	186	187	201	202	198
C. carboxum xylN	124	158	159	170	173	188	189	203	204	199
C. carboxum xylO	125	160	161	172	175	190	191	205	206	200
C. carboxum xylP	126	162	163	174	177	192	193	207	208	201
C. carboxum xylQ	127	164	165	176	179	194	195	209	210	202
C. carboxum xylR	128	166	167	178	181	196	197	211	212	203
C. carboxum xylS	129	168	169	180	183	198	199	213	214	204
C. carboxum xylT	130	170	171	182	185	200	201	215	216	205
C. carboxum xylU	131	172	173	184	187	202	203	217	218	206
C. carboxum xylV	132	174	175	186	189	204	205	219	220	207
C. carboxum xylW	133	176	177	188	191	206	207	221	222	208
C. carboxum xylX	134	178	179	190	193	208	209	223	224	209
C. carboxum xylY	135	180	181	192	195	210	211	225	226	210
C. carboxum xylZ	136	182	183	194	197	212	213	227	228	211
C. carboxum xylA	137	184	185	196	199	214	215	229	230	212
C. carboxum xylB	138	186	187	198	201	216	217	231	232	213
C. carboxum xylC	139	188	189	200	203	218	219	233	234	214
C. carboxum xylD	140	190	191	202	205	220	221	235	236	215
C. carboxum xylE	141	192	193	204	207	222	223	237	238	216
C. carboxum xylF	142	194	195	206	209	224	225	239	240	217
C. carboxum xylG	143	196	197	208	211	226	227	241	242	218
C. carboxum xylH	144	198	199	210	213	228	229	243	244	219
C. carboxum xylI	145	200	201	212	215	230	231	245	246	220
C. carboxum xylJ	146	202	203	214	217	232	233	247	248	221
C. carboxum xylK	147	204	205	216	219	234	235	249	250	222
C. carboxum xylL	148	206	207	218	221	236	237	251	252	223
C. carboxum xylM	149	208	209	220	223	238	239	253	254	224
C. carboxum xylN	150	210	211	222	225	240	241	255	256	225
C. carboxum xylO	151	212	213	224	227	242	243	257	258	226
C. carboxum xylP	152	214	215	226	229	244	245	259	260	227
C. carboxum xylQ	153	216	217	228	231	246	247	261	262	228
C. carboxum xylR	154	218	219	230	233	248	249	263	264	229
C. carboxum xylS	155	220	221	232	235	250	251	265	266	230
C. carboxum xylT	156	222	223	234	237	252	253	267	268	231
C. carboxum xylU	157	224	225	236	239	254	255	269	270	232
C. carboxum xylV	158	226	227	238	241	256	257	271	272	233
C. carboxum xylW	159	228	229	240	243	258	259	273	274	234
C. carboxum xylX	160	230	231	242	245	260	261	275	276	235
C. carboxum xylY	161	232	233	244	247	262	263	277	278	236
C. carboxum xylZ	162	234	235	246	249	264	265	279	280	237
C. carboxum xylA	163	236	237	248	251	266	267	281	282	238
C. carboxum xylB	164	238	239	250	253	268	269	283	284	239
C. carboxum xylC	165	240	241	252	255	270	271	285	286	240
C. carboxum xylD	166	242	243	254	257	272	273	287	288	241
C. carboxum xylE	167	244	245	256	259	274	275	289	290	242
C. carboxum xylF	168	246	247	258	261	276	277	291	292	243
C. carboxum xylG	169	248	249	260	263	278	279	293	294	244
C. carboxum xylH	170	250	251	262	265	280	281	295	296	245
C. carboxum xylI	171	252	253	264	267	282	283	297	298	246
C. carboxum xylJ	172	254	255	266	269	284	285	299	300	247
C. carboxum xylK	173	256	257	268	271	286	287	301	302	248
C. carboxum xylL	174	258	259	270	273	288	289	303	304	249
C. carboxum xylM	175	260	261	272	275	290	291	305	306	250
C. carboxum xylN	176	262	263	274	277	292	293	307	308	251
C. carboxum xylO	177	264	265	276	279	294	295	309	310	252
C. carboxum xylP	178	266	267	278	281	296	297	311	312	253
C. carboxum xylQ	179	268	269	280	283	298	299	313	314	254
C. carboxum xylR	180	270	271	282	285	300	301	315	316	255
C. carboxum xylS	181	272	273	284	287	302	303	317	318	256
C. carboxum xylT	182	274	275	286	289	304	305	319	320	257
C. carboxum xylU	183	276	277	288	291	306	307	321	322	258
C. carboxum xylV	184	278	279	290	293	308	309	323	324	259
C. carboxum xylW	185	280	281	292	295	310	311	325	326	260
C. carboxum xylX	186	282	283	294	297	312	313	327	328	261
C. carboxum xylY	187	284	285	296	299	314	315	329	330	262
C. carboxum xylZ	188	286	287	298	301	316	317	331	332	263
C. carboxum xylA	189	288	289	300	303	318	319	333	334	264
C. carboxum xylB	190	290	291	302	305	320	321	335	336	265
C. carboxum xylC	191	292	293	304	307	322	323	337	338	266
C. carboxum xylD	192	294	295	306	309	324	325	339	340	267
C. carboxum xylE	193	296	297	308	311	326	327	341	342	268
C. carboxum xylF	194	298	299	310	313	328	329	343	344	269
C. carboxum xylG	195	300	301	312	315	330	331	345	346	270
C. carboxum xylH	196	302	303	314	317	332	333	347	348	271
C. carboxum xylI	197	304	305	316	319	334	335	349	350	272
C. carboxum xylJ	198	306	307	318	321	336	337	351	352	273
C. carboxum xylK	199	308	309	320	323	338	339	353	354	274
C. carboxum xylL	200	310	311	322	325	340	341	355	356	275
C. carboxum xylM	201	312	313	324	327	342	343	357	358	276
C. carboxum xylN	202	314	315	326	329	344	345	359	360	277
C. carboxum xylO	203	316	317	328	331	346	347	361	362	278
C. carboxum xylP	204	318	319	330	333	348	349	363	364	279
C. carboxum xylQ	205	320	321	332	335	350	351	365	366	280
C. carboxum xylR	206	322	323	334	337	352	353	367	368	281
C. carboxum xylS	207	324	325	336	339	354	355	369	370	282
C. carboxum xylT	208	326	327	338	341	356	357	371	372	283
C. carboxum xylU	209	328	329	340	343	358	359	373	374	284
C. carboxum xylV	210	330	331	342	345	360	361	375	376	285
C. carboxum xylW	211	332	333	344	347	362	363	377	378	286
C. carboxum xylX	212	334	335	346	349	364	365	379	380	287
C. carboxum xylY	213	336	337	348	351	366	367	381	382	288
C. carboxum xylZ	214	338	339	350	353	368	369	383	384	289
C. carboxum xylA	215	340	341	352	355	370	371	385	386	290
C. carboxum xylB	216	342	343	354	357	372	373	387	388	291
C. carboxum xylC	217	344	345	356	359	374	375	389	390	292
C. carboxum xylD	218	346	347	358	361	376	377	391	392	293
C. carboxum xylE	219	348	349	360	363	378	379	393	394	294
C. carboxum xylF	220	350	351	362	365	380	381	395	396	295
C. carboxum xylG	221	352	353	364	367	382	383	397	398	296
C. carboxum xylH	222	354	355	366	369	384	385	399	400	297
C. carboxum xylI	223	356	357	368	371	386	387	401	402	298
C. carboxum xylJ	224	358	359	370	373	388	389	403	404	299
C. carboxum xylK	225	360	361	372	375	390	391	405	406	300
C. carboxum xylL	226	362	363	374	377	392	393	407	408	301
C. carboxum xylM	227	364	365	376	379	394	395	409	410	302
C. carboxum xylN	228	366	367	378	381	396	397	411	412	303
C. carboxum xylO	229	368	369	380	383					

Figure 1 continued

B. subtilis xyla	271	285	286	300	301	315	316	330	331	345	346	360
B. circulans xyla	---	---	---	---	---	---	---	---	---	---	---	---
B. stearothermophilus xyla	---	---	---	---	---	---	---	---	---	---	---	---
A. caviae xyla	---	---	---	---	---	---	---	---	---	---	---	---
H. turcicum xyl1	---	---	---	---	---	---	---	---	---	---	---	---
A. plasi xyl	---	---	---	---	---	---	---	---	---	---	---	---
S. commune xyla	---	---	---	---	---	---	---	---	---	---	---	---
T. languiensis xyla	---	---	---	---	---	---	---	---	---	---	---	---
C. carbonum xyl2	---	---	---	---	---	---	---	---	---	---	---	---
H. insolens xyl1	---	---	---	---	---	---	---	---	---	---	---	---
M. grisea xyl12	---	---	---	---	---	---	---	---	---	---	---	---
C. gracile cypA	---	---	---	---	---	---	---	---	---	---	---	---
T. reesei xyl2(2)	---	---	---	---	---	---	---	---	---	---	---	---
T. reesei xyl2(271 xyl2)	---	---	---	---	---	---	---	---	---	---	---	---
T. harizianum xylD	---	---	---	---	---	---	---	---	---	---	---	---
C. gracile cypB	---	---	---	---	---	---	---	---	---	---	---	---
A. niger xyl1	---	---	---	---	---	---	---	---	---	---	---	---
A. niger xyl2	---	---	---	---	---	---	---	---	---	---	---	---
Streptomyces sp xyl1	---	---	---	---	---	---	---	---	---	---	---	---
S. thermocycloolacensis xylB	---	---	---	---	---	---	---	---	---	---	---	---
S. viridosporus T7A svxA	---	---	---	---	---	---	---	---	---	---	---	---
C. packae xyl1	---	---	---	---	---	---	---	---	---	---	---	---
A. oryzae xylG1	---	---	---	---	---	---	---	---	---	---	---	---
C. purpurea xyl1	---	---	---	---	---	---	---	---	---	---	---	---
C. mixtus xyl1	---	---	---	---	---	---	---	---	---	---	---	---
P. fluorescens cellulosa xyl1	---	---	---	---	---	---	---	---	---	---	---	---
S. glaucus xyl1	---	---	---	---	---	---	---	---	---	---	---	---
A. niger xyl1	---	---	---	---	---	---	---	---	---	---	---	---
A. tubigenensis xyl1	---	---	---	---	---	---	---	---	---	---	---	---
P. purpogenum xylB	---	---	---	---	---	---	---	---	---	---	---	---
Cryptococcus sp S-2 xyl-CS2	---	---	---	---	---	---	---	---	---	---	---	---
B. pumilus xylA(1)	---	---	---	---	---	---	---	---	---	---	---	---
B. pumilus xylA(2)	---	---	---	---	---	---	---	---	---	---	---	---
C. acetobutylicum xylB	---	---	---	---	---	---	---	---	---	---	---	---
Bacillus sp 41M-1 xylJ	---	---	---	---	---	---	---	---	---	---	---	---
C. thermophilus xylA	---	---	---	---	---	---	---	---	---	---	---	---
P. multivesiculatum xyl1	---	---	---	---	---	---	---	---	---	---	---	---
B. albus xyla	---	---	---	---	---	---	---	---	---	---	---	---
Caldicellulosiruptor sp xylD	---	---	---	---	---	---	---	---	---	---	---	---
D. thermophilum xynB	---	---	---	---	---	---	---	---	---	---	---	---
B. stearothermophilus xyla	---	---	---	---	---	---	---	---	---	---	---	---
P. stipitis xyla	---	---	---	---	---	---	---	---	---	---	---	---

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/IB 02/03797

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/24 C12N9/42

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the lists searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, SEQUENCE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	the whole document ---	1-23
X	WO 00 39289 A (SOERENSEN JENS FRISBAEK ;DANISCO (DK); SIBBESEN OLE (DK)) 6 July 2000 (2000-07-06)	7-13,17, 20-23
Y	page 19, line 13 page 63, line 25 - line 28; claims 1-7; figure 20; table 14 ---	1-23
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	column 3 -column 20; claim 3; figures 1-5 ---	
X	WO 00 68396 A (XENCOR INC) 16 November 2000 (2000-11-16)	7-13,17, 20,21
	examples 2-5 ---	
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

International Application No.

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